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ARD1 and NAT1 proteins form a complex that has N-terminal acetyltransferase activity

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Two yeast genes, *ARD1* and *NAT1*, are required for the expression of an N-terminal protein acetyltransferase. This activity is required for full repression of the silent mating type locus *HML*, for sporulation, and for entry into G_0. While the *NAT1* gene product is thought to be the catalytic subunit of the enzyme, the role of the ARD1 protein has remained unclear. We have used epitope tagged derivatives of ARD1 and NAT1 to provide biochemical evidence for the formation of an ARD1–NAT1 complex, and to show that both proteins are required for the N-terminal acetyltransferase activity. We also present evidence for the formation of ARD1–ARD1 homodimers. Deletion analysis suggests that the C-terminal region of ARD1 may be involved in the formation of both ARD1–ARD1 and ARD1–NAT1 complexes.

Key words: protein modification/protein–protein interactions/yeast

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Materials and methods

Yeast strains and media

The genotypes and sources of *S.cerevisiae* used in this work are as follows: M107-8B, *MATα his3 leu2 ura3 trp1 can1 ard1::HIS3* (M.Whiteway); M107-6C, *MATα his3 leu2 ura3 can1* (M.Whiteway); 9-2B, *MAT***a** *his3 leu2 ura3 can1 ard1::URA3* (M.Whiteway); 9-3C, *MAT***a** *his3 leu2 ura3 can1* (M.Whiteway); W303-1a, *MAT***a** *his3 leu2 ura3 can1* (R.Sternglanz);

and AMR7, *MAT***a** *his3 leu2 ura3 can1 nat1::URA3* (R.Sternglanz). SD contained 0.67% Difco yeast nitrogen base without amino acids, 2% glucose, and amino acids essential for auxotrophic strains as described by Sherman *et al.* (1981). SD-galactose was the same as SD except that galactose was added instead of glucose.

Plasmids

The chimeric proteins used in this study are diagrammed in Figure 1. Wild-type ARD1 protein and all of the following hybrid proteins were overexpressed by placing the corresponding gene under the control of the *DED1* promoter (Hope and Struhl, 1986) on a multicopy plasmid. The lexA−ARD fusion protein was made by fusing the DNA binding domain of lexA (amino acids 1−87, encoded by a *Hin*dIII−*Xmn*I fragment of plasmid pRB480 (provided by R.Brent) in frame to the entire ARD1 coding sequence. The related ARD25 protein consists of the same lexA DNA binding domain fused to seven amino acids (PTLEDPH) derived from a polylinker sequence, followed by a C-terminal fragment of ARD1 (amino acids 166−238). ARD34 is similar to ARD25 except that the last 11 amino acids of ARD1 (amino acids 228−238) were replaced by a c-myc epitope (DMEQKLISEEDLN) derived from the plasmid pUCmyc (provided by B.Bartel). ARDmyc contains amino acids 1−227 of ARD1 followed by the same c-myc epitope.

The ARDdra and M−ARD proteins begin with a short segment of lacI (amino acids 318−346, derived from pUB23, provided by A.Varshavsky) followed by the c-myc epitope, with transcription under the control of the *GAL10* promoter (Guarente *et al.*, 1982) as previously described (Park *et al.*, 1992). This lacI−myc sequence was fused in frame to the N-terminal amino acids 1−194 of the ARD1 coding sequence to generate ARDdra, or to the entire *ARD1* gene to generate M−ARD.

Wild-type NAT1 was expressed under the control of the ADH1 promoter on plasmid pJM124 (Mullen *et al.*, 1989; provided by R.Sternglanz). The epitope tagged fluNAT protein begins with the lacI segment described above, followed by a hemagglutinin epitope (Park *et al.*, 1992), followed by the entire NAT1 sequence. The epitope tagged NATmyc protein was generated by PCR (Saiki *et al.*, 1985) modification of NAT1 and contains the entire coding sequence of *NAT1* followed by 11 amino acids (MEQKLISEEDL) of a c-myc epitope (Evans *et al.*, 1985).

Transcriptional activation experiments were done in strains bearing the multicopy plasmid YEp21-Sc3423 (Hope and Struhl, 1986; provided by K.Struhl) which carries the lexA operator upstream of a *lacZ* target gene.

Immunological methods

Cells carrying appropriate plasmids were grown in selective medium (SD or SD-galactose medium) until they reached exponential growth phase. 10 ml cultures were harvested and resuspended in 500 μl of SD-met medium containing 100 μCi of [^{35}S]methionine (ICN) and labeled for 20 min at room temperature with shaking. Cells were harvested and resuspended in 500 μl of IP buffer (150 mM NaCl, 50 mM Tris 7.5, 5 mM EDTA and 1% Triton X-100) containing protease inhibitors (2 μg/ml pepstatin A, 10 μg/ml aprotonin, 5 μg/ml phosphoramidon, 20 μg/ml chymostatin, 5 μg/ml E-64 and 1 mM PMSF). Cells were transferred to Eppendorf tubes containing 0.4 ml of chilled glass beads and were disrupted by vortexing for 3 min. Lysates were centrifuged for 15 min at 12 000 *g* at 4°C, and the amount of acid-insoluble ^{35}S in the supernatant was determined. Equal amounts of total acid-insoluble ^{35}S were processed for immunoprecipitation by incubating with an excess of the indicated antibody for 1 h. The antibody bound proteins were precipitated with Trisacryl−protein A beads (Pierce) and the pellets were washed three times with IP buffer containing 0.1% SDS. The immunoprecipitates were either analyzed by discontinous PAGE with subsequent fluorography or assayed for N-terminal acetyltransferase activity.

Western blot analysis of immunoprecipitates was done by electroblotting proteins fractionated by SDS−PAGE onto a PVDF membrane (Millipore) in 0.19 M glycine, 0.05 M Tris base, 0.01% SDS and 20% MeOH at 4°C. After transfer, membranes were blocked with 20% FCS in 1×PBS for 1 h, incubated with appropriate antibodies overnight at 4°C, washed and visualized using alkaline phosphatase-conjugated secondary antibodies (Promega) following the manufacturer's instructions.

Glycerol gradient

Glycerol gradients (10−40%) were prepared by mixing in a gradient mixer 5.5 ml of 10% glycerol in IP buffer (without Triton X-100 and with protease inhibitors) and 6.2 ml 40% glycerol in the same buffer. Cell extracts were prepared as for immunoprecipitation and 100 μl of each was loaded on top of each gradient. Samples thus prepared were centrifuged at 200 000 *g* at 4°C for 35 h, and were collected in ~300 μl fractions. As size markers, catalase (236 kDa, Sigma) and aldolase (160 kDa, Sigma) were mixed with samples prior to loading onto gradients. Fractions 10−14 from the bottom of the gradient were assayed for catalase and fractions 15−22 for aldolase, following instructions provided by the manufacturer. Every fraction was subjected to immunoprecipitation using anti-myc antibody as described above.

N-terminal acetyltransferase assay

The enzyme assay was carried out on immunoprecipitates as described (Lee *et al.*, 1988) with minor modifications. We only assayed immunoprecipitates since crude extracts contain other acetyltranferase activities and non-specific hydrolases that interfere with the assay. An assay mixture contains the enzyme (immunoprecipitate), 20 μM ACTH (1−24) and 6.8 μM [^{3}H]acetyl CoA (3.18 Ci/mmol, Amersham) in 75 mM Tris pH 8.2, 150 mM NaCl, 0.1 mM EDTA and 2 mM β-mercaptoethanol. The assay mixture was incubated for 1 h at room temperature. The enzyme activities were measured by counting the amount of radioactivity present in ACTH after collecting the substrate on glass fiber filters.

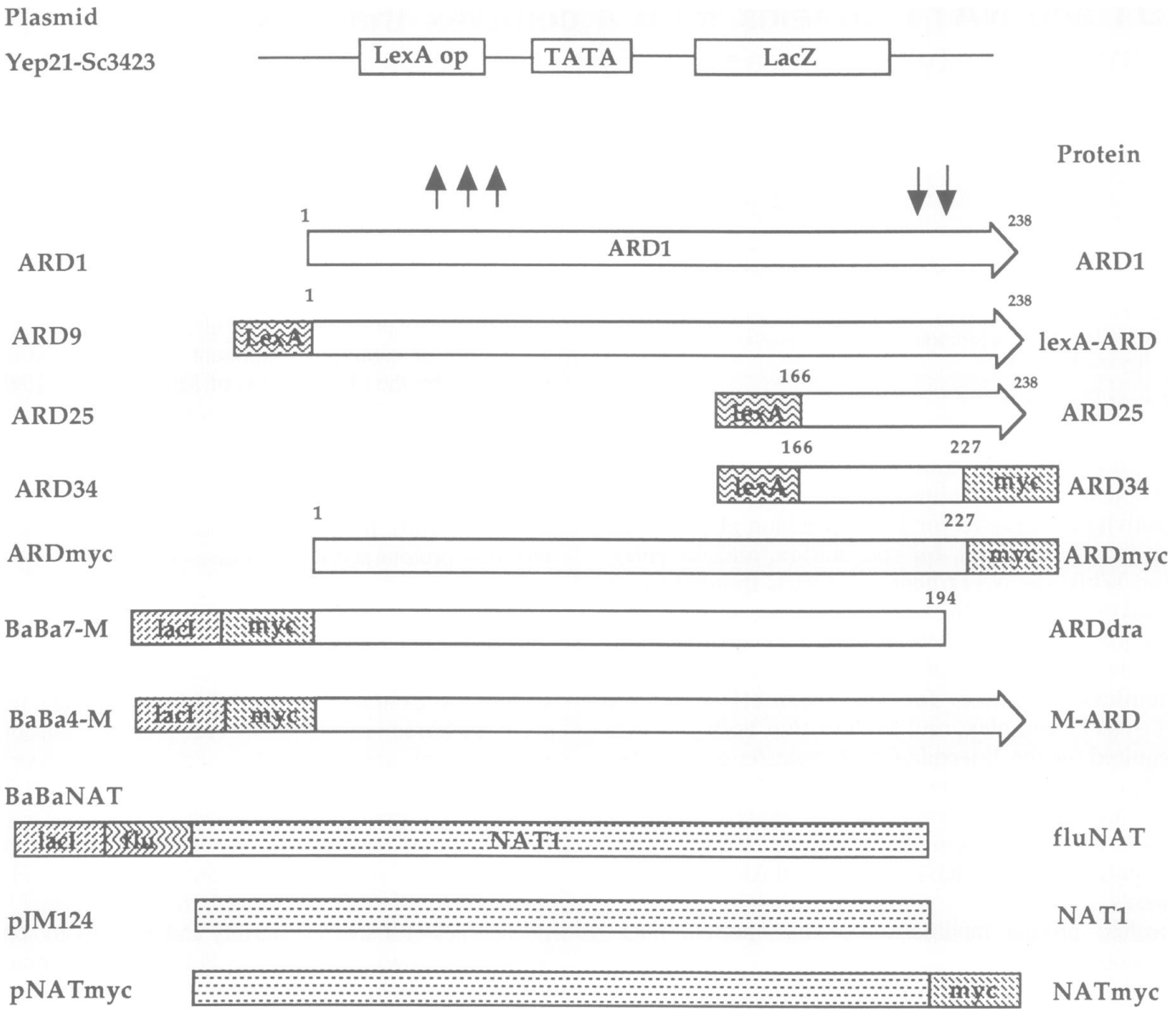

Fig. 1. Schematic diagram of ARD1 and NAT1 derived constructs. Arrows pointing up indicate the location of the stretch of negatively charged amino acids; arrows pointing down indicate the region of the ARD1 protein with sequence similarity to the homeodomain. LexA is the lexA DNA binding domain; myc represents the epitope recognized by the monoclonal antibody 9E10 (Evans *et al.*, 1985); flu is the epitope recognized by the monoclonal antibody 12CA5 (Field *et al.*, 1988); and lacI represents a segment of the *lacI* gene (Park and Szostak, 1992).

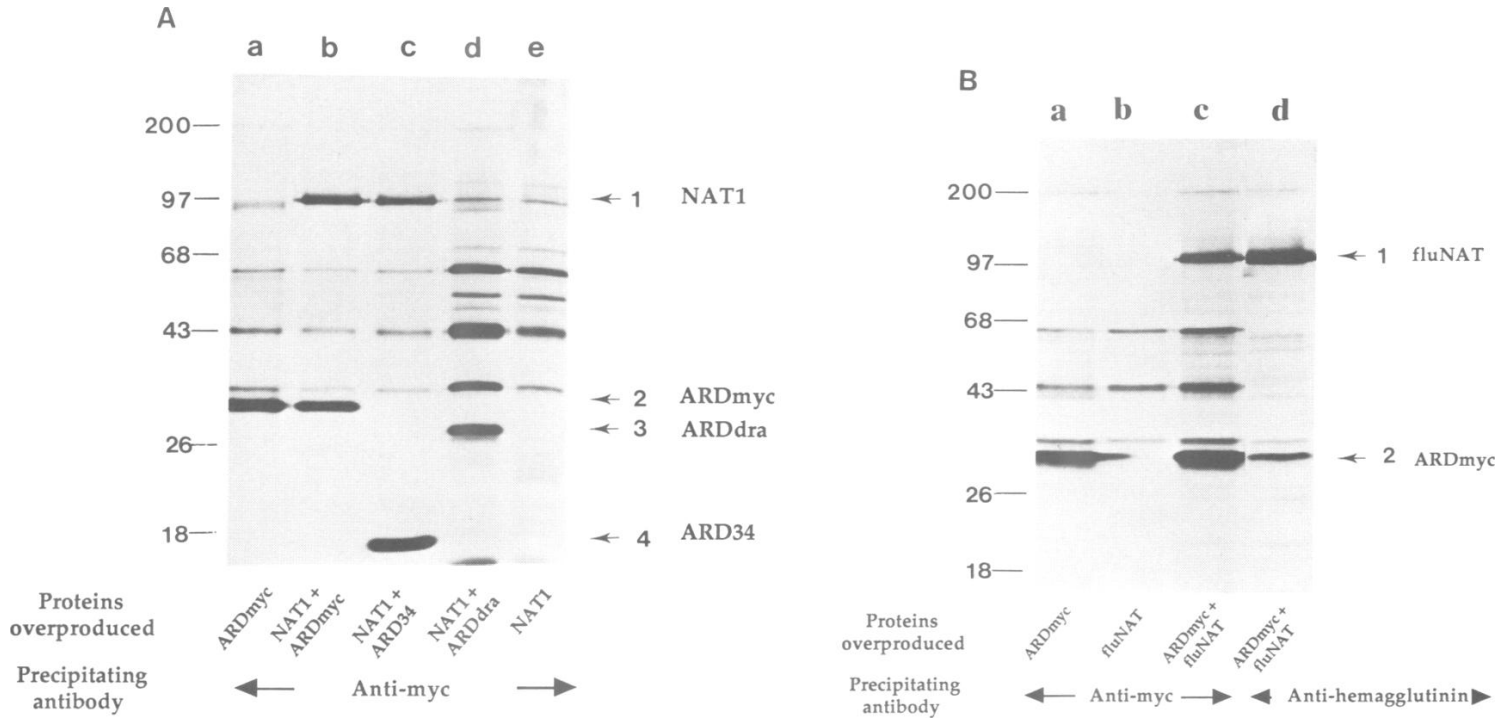

Fig. 2. Co-immunoprecipitation of ARD1 and NAT1 proteins. (**A**) Derivatives of strain 9-3C overexpressing ARDmyc (lane a), NAT1 and ARDmyc (lane b), NAT1 and ARD34 (lane c), NAT1 and ARDdra (lane d) and NAT1 (lane e) were labeled with [^{35}S]methionine. Extracts made from these cultures were immunoprecipitated with the anti-myc monoclonal antibody. The immunoprecipitates were analyzed on 15% SDS−PAGE and subjected to fluorography. Band 1 corresponds to NAT1, band 2 to ARDmyc, band 3 to ARDdra and band 4 to ARD34. More immunoprecipitate was loaded in lanes d and e than in lanes a, b and c (compare intensities of 43 kDa background band). (**B**) Derivatives of strain W303-1a overexpressing either ARDmyc (lane a), fluNAT (lane b), or both ARDmyc and fluNAT (lanes c and d), were labeled with [^{35}S]methionine. Extracts made from these cultures were immunoprecipitated with the anti-myc monoclonal antibody (lanes a−c) or the anti-hemagglutinin monoclonal antibody (lane d). The immunoprecipitates were fractionated by 15% SDS−PAGE and subjected to fluorography. Band 1 corresponds to fluNAT, and band 2 to ARDmyc. Plasmids used to overproduce the proteins indicated are shown in Figure 1.

Table I. β-galactosidase activities induced by lexA fusion proteins

LexA fusions	β-galactosidase activity (units)	
	ARD1	ard1
lexA−ARD	68	96
ARD25	35	12
ARD34	24	4
None	1	1

A unit is defined by OD420×1000/(min)(OD600)(ml). Strains used for the experiment are M107-6B (ARD1) and M107-8B (ard1). The *lacZ* gene was placed downstream of a lexA operator and carried on plasmid YEp21-Sc3423 (Hope and Struhl, 1986). Plasmids expressing the lexA fusion proteins are shown in Figure 1.

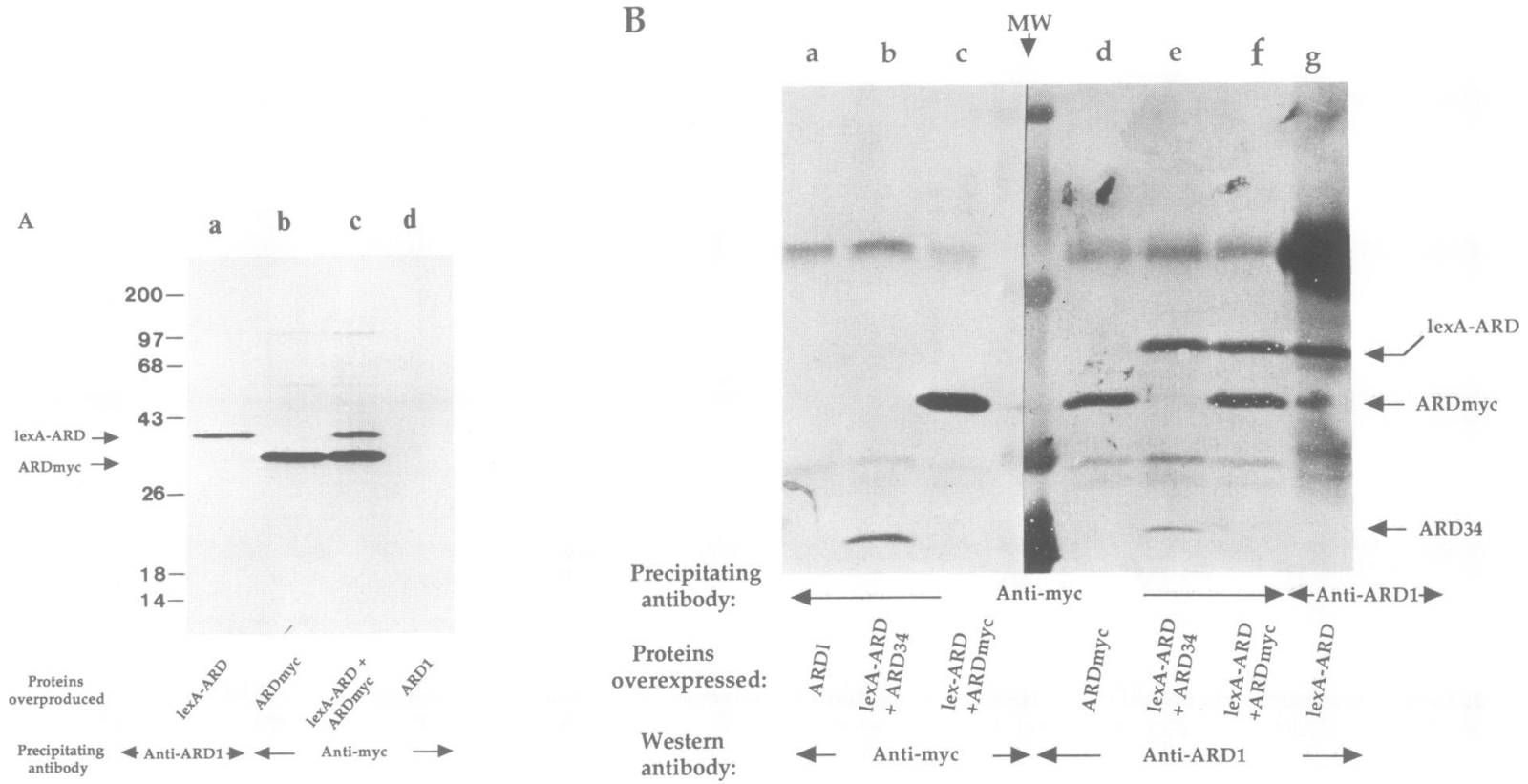

Fig. 3. Formation of ARD1 dimers. (**A**) Co-immunoprecipitation of two forms of ARD1. Derivatives of strain 9-3C overexpressing lexA−ARD (lane a), ARDmyc (lane b), lexA−ARD and ARDmyc (lane c), and wild-type ARD1 (lane d) were labeled with [^{35}S]methionine. Extracts made from these cells were immunoprecipitated with either anti-ARD1 antisera (lane a) or anti-myc monoclonal antibody (lanes b−d). (**B**) Western blot analysis of immunoprecipitates. Derivatives of strain 9-3C overexpressing ARD1 (lane a), lexA−ARD and ARD34 (lanes b and e), lexA−ARD and ARDmyc (lanes c and f), ARDmyc (lane d), or lexA−ARD (lane g) were labeled with [^{35}S]methionine. Extracts made from these cultures were immunoprecipitated with either the anti-myc monoclonal antibody (lanes a−f) or anti-ARD1 antisera (lane g). The precipitates were fractionated by 15% SDS−PAGE, blotted onto PVDF membrane, and probed with either the anti-myc antibody (lanes a−c) or anti-ARD1 antibody (lanes d−g). Plasmids used to overproduce the proteins indicated are shown in Figure 1.

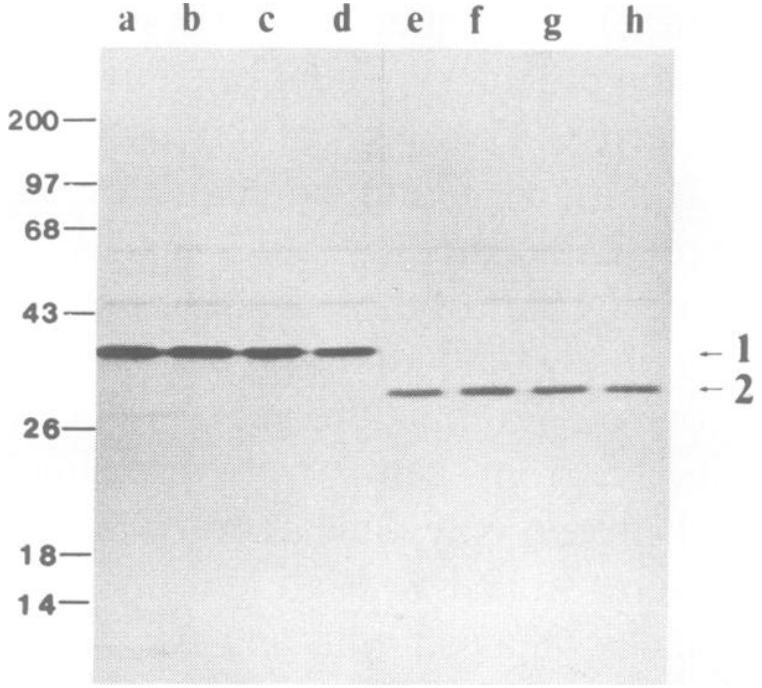

Fig. 4. Comparison of stabilities of M−ARD and ARDdra. Derivatives of strain (9-3C) expressing M−ARD (lanes a−d), or ARDdra (lanes e−h) were pulse-labeled for 5 min and chased for 0 (lanes a and e), 10 (lanes b and f), 30 (lanes c and g) and 60 (lanes d and h) min. Band 1 corresponds to M−ARD and band 2 to ARDdra. Plasmids used to overproduce the proteins indicated are shown in Figure 1.

Table II. N-terminal acetyltransferase activities

Strain genotype	Overexpressed protein(s)	Extract	Activity in immunoprecipitate (k.c.p.m.)
WT	ARDmyc + NAT1	A	1700–10 000
WT	ARD1 + NATmyc	B	17–106
nat1	ARDmyc	C	<1
ard1	NATmyc	D	<1
WT	NAT1	E	<1
		C+E	13–112

In all cases, immunoprecipitates were obtained by using an anti-myc antibody. Equal fractions of immunoprecipitates obtained from 8 ml cultures were assayed for N-terminal acetyltransferase activity as described in Materials and methods. WT, W303-1a; *nat1*, AMR7 and *ard1*, 9-2B.

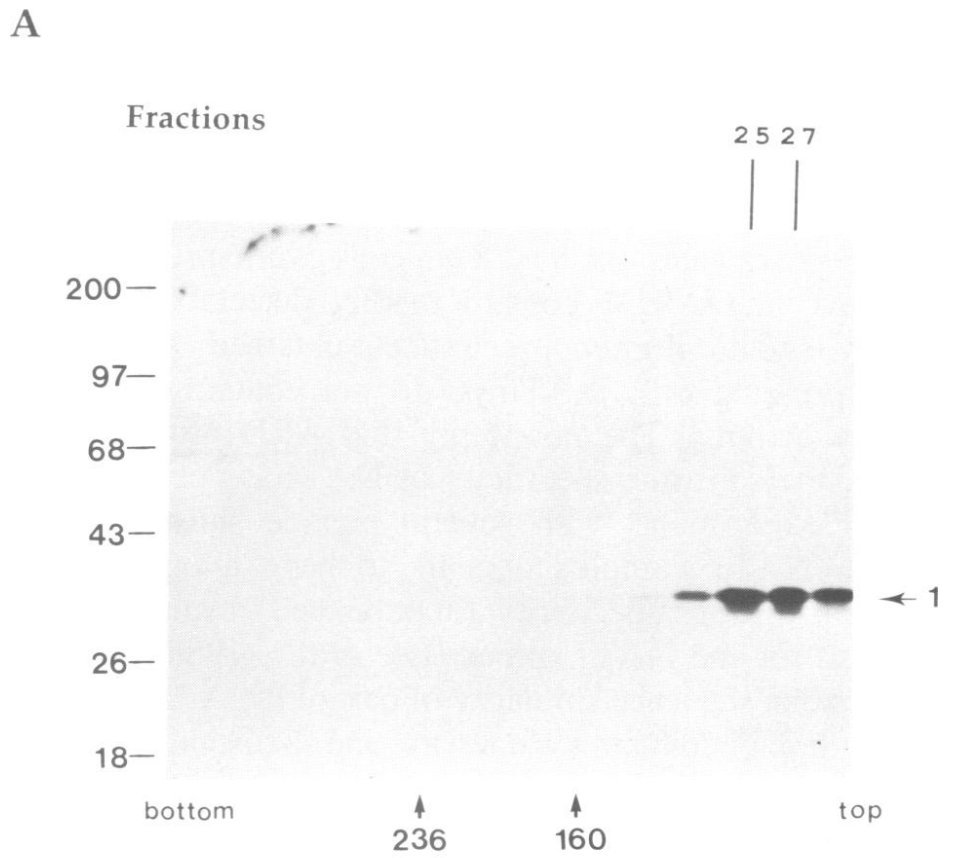

Fig. 5. Glycerol gradient fractionation of cell extracts. Derivatives of strains (9-3C) overexpressing ARDmyc alone (**A**) or ARDmyc and NAT1 (**B**) were labeled with [^{35}S]methionine. Extracts made from these cells were separated on 10−40% glycerol gradients. Odd-numbered fractions were immunoprecipitated with anti-myc antibody and analyzed by 15% SDS−PAGE. Band 1 corresponds to ARDmyc and band 2 to NAT1. Positions of molecular weight markers, catalase (236 kDa) and aldolase (160 kDa), are shown. Even-numbered fractions were used for N-terminal acetyltransferase assays (shown above gel B) after immunoprecipitation with anti-myc antibody.

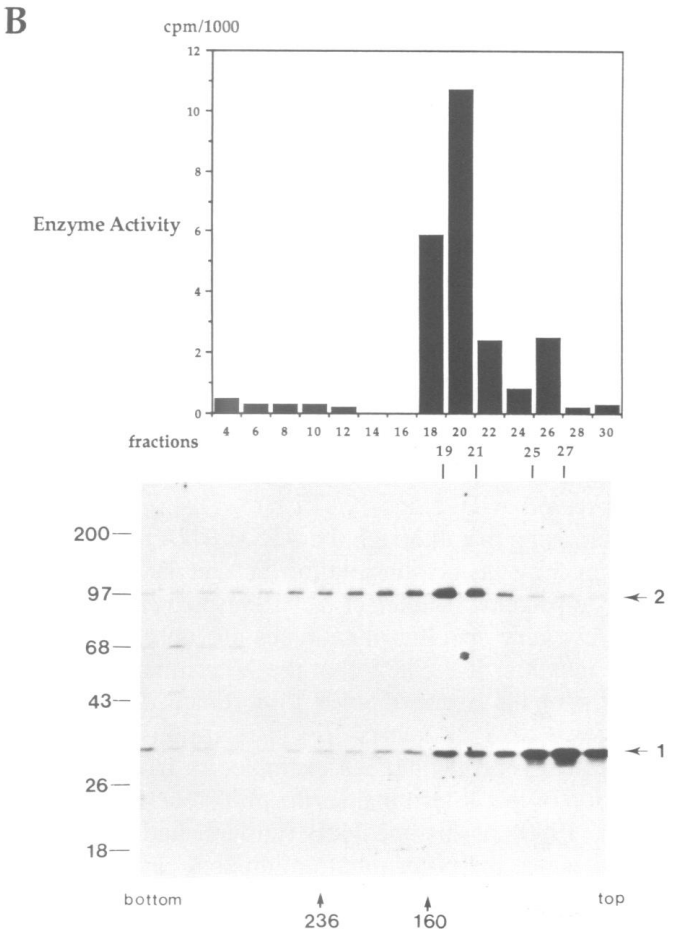

Fig. 5. Glycerol gradient fractionation of cell extracts. Derivatives of strains (9-3C) overexpressing ARDmyc alone (**A**) or ARDmyc and NAT1 (**B**) were labeled with [^{35}S]methionine. Extracts made from these cells were separated on 10–40% glycerol gradients. Odd-numbered fractions were immunoprecipitated with anti-myc antibody and analyzed by 15% SDS–PAGE. Band 1 corresponds to ARDmyc and band 2 to NAT1. Positions of molecular weight markers, catalase (236 kDa) and aldolase (160 kDa), are shown. Even-numbered fractions were used for N-terminal acetyltransferase assays (shown above gel B) after immunoprecipitation with anti-myc antibody.